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TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Application Number	09/835,759
Filing Date	April 16, 2001
First Named Inventor	Emilio Barbera-Guillem
Art Unit	1642
Examiner Name	David J. Blanchard
Total Number of Pages in This Submission: 37 Pages	Attorney Docket Number: 26983-98

ENCLOSURES *(Check all that apply)*

- [✓] Fee Transmittal Form
  - [✓] Fee Attached
- [ ] Amendment/Reply
  - [ ] After Final
  - [ ] Affidavits/declaration(s)
- [ ] Extension of Time Request
- [ ] Express Abandonment Request
- [ ] Information Disclosure Statement
- [ ] Certified Copy of Priority Document(s)
- [ ] Response to Missing Parts/ Incomplete Application
  - [ ] Response to Missing Parts under 37 CFR 1.52 or 1.53
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Firm or Individual name	Todd L. Sladek, Reg. No. 53,768
Signature	Todd L. Sladek
Date	June 22, 2005

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FEE TRANSMITTAL for FY 2004

Effective 10/01/2003. Patent fees are subject to annual revision.

☑ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 250.00

Complete if Known	
Application Number	09/835,759
Filing Date	April 16, 2001
First Named Inventor	Emilio Barbera-Guillem
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METHOD OF PAYMENT *(check all that apply)*

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Deposit Account Number: 02-2051

Deposit Account Name: Benesch, Friedlander, C..

The Director is authorized to: *(check all that apply)*

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FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1001	770	2001	385	Utility filing fee	
1002	340	2002	170	Design filing fee	
1003	530	2003	265	Plant filing fee	
1004	770	2004	385	Reissue filing fee	
1005	160	2005	80	Provisional filing fee	

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2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

		Extra Claims		Fee from below	Fee Paid
Total Claims		-20** =		X	=
Independent Claims		- 3** =		X	=
Multiple Dependent					=

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
1202	18	2202	9	Claims in excess of 20
1201	86	2201	43	Independent claims in excess of 3
1203	290	2203	145	Multiple dependent claim, if not paid
1204	86	2204	43	** Reissue independent claims over original patent
1205	18	2205	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($)

**or number previously paid, if greater; For Reissues, see above

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1051	130	2051	65	Surcharge - late filing fee or oath	
1052	50	2052	25	Surcharge - late provisional filing fee or cover sheet	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for *ex parte* reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	420	2252	210	Extension for reply within second month	
1253	950	2253	475	Extension for reply within third month	
1254	1,480	2254	740	Extension for reply within fourth month	
1255	2,010	2255	1,005	Extension for reply within fifth month	
1401	330	2401	165	Notice of Appeal	
1402	330	2402	165	Filing a brief in support of an appeal	250.00
1403	290	2403	145	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive - unavoidable	
1453	1,330	2453	665	Petition to revive - unintentional	
1501	1,330	2501	665	Utility issue fee (or reissue)	
1502	480	2502	240	Design issue fee	
1503	640	2503	320	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR 1.17(q)	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	770	2809	385	Filing a submission after final rejection (37 CFR 1.129(a))	
1810	770	2810	385	For each additional invention to be examined (37 CFR 1.129(b))	
1801	770	2801	385	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	

Other fee (specify) _____

*Reduced by Basic Filing Fee Paid

SUBTOTAL (3) ($) 250.00

SUBMITTED BY

(Complete *(if applicable)*)

Name *(Print/Type)*	Todd L. Sladek	Registration No. *(Attorney/Agent)*	53,768	Telephone	216-363-4500
Signature	Todd L. Sladek			Date	June 22, 2005

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	:	Emilio Barbera-Guillem	Examiner	:	Blanchard, David J.
Application No.	:	09/835,759	Group Art	:	1642
Filing Date	:	April 16, 2001	Docket No.	:	26983-98
Confirmation No.	:	5302			
Title	:	**VACCINE AND IMMUNOTHERAPY FOR SOLID NONLYMPHOID TUMOR AND RELATED DYSREGULATION**			

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United States Patent and Trademark Office
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APPEAL BRIEF

Sir/Madam:

The following Appeal Brief is submitted pursuant to the Notice of Appeal filed April 22, 2005 in the above-identified application. The Appeal Brief is filed within two months of the filing date of the Notice of Appeal and, therefore, is timely filed. This is an Appeal from the decision of the Examiner mailed January 26, 2005, finally rejecting claims 1-13 and 69-115. The Appeal Brief is accompanied by the fee set forth in 37 CFR § 41.20(b)(2), as stated in the accompanying Fee Transmittal Form.

TABLE OF CONTENTS

TABLE OF AUTHORITIES

Cases

Statutes

Other Authorities

I. REAL PARTY IN INTEREST

BioCrystal, Ltd., 575 McCorkle Boulevard, Westerville, Ohio 43082, is the assignee of the present application and the real party in interest.

II. RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences.

III. STATUS OF CLAIMS

Claims 1-5, 7-12, 69-73, 77, 78, 80-86, 90, 92-96, 100, 102-108, 112, 114 and 115 are pending and are on appeal (see Section IX of this document, Claims Appendix).

Claims 1, 2 and 7-10 were rejected under 35 U.S.C. § 102(b).

Claims 1-5, 7-12, 69-73, 77, 78, 80-86, 90, 92-96, 100, 102-108, 112, 114 and 115 were rejected under 35 U.S.C. § 103(a).

Claims 70, 71, 73, 78, 82-84, 86, 89, 90, 93, 94, 96, 100, 105, 106, 108 and 112 were rejected under 35 U.S.C. § 112, first paragraph.

Claims 14-68 were canceled.

Additionally, Appellant notes claims that were finally rejected in the Office Action of January 26, 2005, but are not being appealed herein. These include claims 6, 13, 79, 91, 101, 113, rejected under § 112, second paragraph, and claims 74-76, 87-89, 97-99 and 109-111, objected to under 37 CFR § 1.75(c). These claims are hereby canceled to narrow the issues under consideration in this appeal.

IV. STATUS OF AMENDMENTS

No amendments were filed subsequent to the final Office Action, mailed January 26, 2005.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The pending claims are drawn to compositions that include two components. The first component is an immunotherapeutic composition for effecting B cell depletion (claims 1 and 9), one such composition being a monoclonal antibody having binding specificity for CD22 (claims 69, 82, 92 and 104). The second component is a tumor-associated antigen capable of inducing a

cell mediated immune response (claims 1, 9, 69, 82, 92 and 104). Additional dependent claims add the following to the composition: anti-B cell agent (claims 4, 11, 78, 90 and 100), additional antibodies (claims 5 and 12), and immunomodulator and/or pharmaceutically acceptable carrier (claims 2, 10, 70, 72, 73, 83-86, 93-96 and 105-108). Claims 3 and 77 specify that the composition is contained in a solid phase implant. The claimed compositions are useful for producing cellular immune responses specific for tumors.

It should be noted that, early in prosecution of this application, Appellant was subjected to a restriction requirement and was also required to elect a species of the immunotherapeutic composition for effecting B cell depletion (see Office Action mailed November 11, 2003). In a response entered February 5, 2004, Appellant elected to prosecute claims drawn to the composition described above, and also elected a species of the immunotherapeutic composition comprising a monoclonal antibody specific for CD22 (also called LL2). Therefore, as stated by the Office, most recently in the final Office Action mailed January 26, 2005 (page 2), "the claims are being examined to the extent that the affinity ligand is a monoclonal antibody specific for CD22 (LL2)..." CD22 is an antigen on the surface of B cells. This is important for examination of claims 1 and 9, since these claims do not explicitly recite a monoclonal antibody specific for CD22.

The conceptual basis for the claimed composition is that cell mediated immunity, as opposed to humoral, or antibody mediated immunity, is particularly important for anti-tumor activity in the body. An overactive humoral, or protumor, immune response may actually promote tumor progression (see specification page 7, lines 9-12). It is known that the prevalence of a particular type of CD4+ T cell is one factor influencing the type of immune response, cellular or humoral, that develops in response to an antigen. CD4+ T cells of a type called "TH1" secrete a panel of cytokines that generally support cell mediated immunity through interactions with other cells. CD4+ T cells of a type called "TH2" secrete a panel of cytokines that generally support a humoral immune response through interactions with other cells. Both TH1 and TH2 cells arise from TH0 cells. Therefore, a stimulus that pushes the immune system toward cell mediated immunity may be said to promote a TH1 response. A stimulus that pushes the immune system toward humoral, or antibody mediated immunity may be said to promote a TH2 response. The claimed composition generally influences the immune system toward cell mediated immunity promoting a TH1 response by: i) using the immunotherapeutic composition

depleting B cells (B cells produce antibodies; antibodies mediate humoral immunity), and ii) the tumor-associated antigen inducing a cell mediated immune response.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

1. Claims 1 (independent), 2, 7 and 8 (depend from claim 1), 9 (independent) and 10 (depend from claim 9) under 35 U.S.C. § 102(b) using Noguchi et al. (Proc. Natl. Acad. Sci. USA 92:2219-2223, 1995; "Noguchi") as evidenced by Trinchieri (Immunology Today 14:335-338, 1993; "Trinchieri").

2. Claims 1 (independent), 2-5, 7 and 8 (depend from claim 1), 9 (independent), 10-12 (depend from claim 9), 69-73, 77, 78, 80-86, 90, 92-96, 100, 102-108, 112, 114 and 115 were rejected under 35 U.S.C. § 103(a) using Apostolopoulos et al. (Vaccine 14:930-938, 1996; "Apostolopoulos") in view of Tachibana et al. (Tokai J. Exp. Clin. Med. 8:455-463, 1983; "Tachibana"), Trinchieri, Parkhouse et al. (Current Topics in Microbiology and Immunology 182:331-335, 1992) and Wang (U.S. Pat. No. 5,939,380).

3. Claims 70, 71, 73, 78, 82-84, 86, 89, 90, 93, 94, 96, 100, 105, 106, 108 and 112 were rejected under 35 U.S.C. § 112, first paragraph as failing to comply with the written description requirement.

VII. ARGUMENT

A. Claims 1, 2 and 7-10 Are Patentable Over Noguchi As Evidenced By Trinchieri

Claims 1, 2 and 7-10 are not anticipated by Noguchi as evidenced by Trinchieri under 35 U.S.C. § 102(b).

1. Brief Discussion of Noguchi and Trinchieri References

The study described in Noguchi discloses a composition that includes interleukin-12 (IL-12) and a nonamer p53 peptide (peptide of 9 amino acids in length) in QS-21 adjuvant.

Trinchieri is a review article disclosing that IL-12 is one of a number of substances that promote TH0 cells to become TH1 cells, and inhibit TH0 cells from becoming TH2 cells.

2. Argument

Appellant's claims 1 and 9 recite an immunotherapeutic composition for effecting B cell depletion. Based on Appellant's species election, as described earlier, this immunotherapeutic composition is being examined to the extent that the immunotherapeutic composition is a monoclonal antibody specific for CD22.

The Patent Office stated (see Office Action mailed May 20, 2004; page 14) that the IL-12 of the Noguchi composition is to be interpreted as an effector of B cell depletion. The Patent Office based this assertion on Figures 1 and 2 of Trinchieri. Additionally, the nonamer p53 peptide of the Noguchi composition is interpreted to be a tumor-associated antigen. Therefore, the Patent Office stated that the composition of Noguchi anticipates Appellant's claimed composition.

The Patent Office noted that Figure 1 of Trinchieri (page 336) illustrates that IL-12 acts on the cell differentiation/selection pathways whereby TH0 cells become either TH1 or TH2 cells. Figure 1 illustrates that IL-12 promotes TH0 cells into becoming TH1 cells (TH1 cells promote cell mediated immunity). Figure 1 also illustrates that IL-12 inhibits TH0 cells from becoming TH2 cells (TH2 cells promote humoral immunity). The rationale of the Patent Office was that, because IL-12 inhibits humoral immunity, IL-12 is an "effector of B cell depletion" as recited in Appellant's claims (Office Action mailed May 20, 2004, page 14; final Office Action mailed January 26, 2005, pages 6-7).

With respect to this characterization of IL-12 as an effector of B cell depletion, the Patent Office is incorrect. First, Appellant notes that Figure 1 of Trinchieri does not show IL-12 acting on B cells. In fact, B cells are not even shown in Figure 1. Second, Appellant has searched the literature and found references showing that B cells have cell surface receptors for IL-12. One such reference is Metzger et al., Ann. NY Acad. Sci 795:100-115, 1996 ("Metzger"; a copy of the abstract of this reference is in the Evidence Appendix beginning on page 25 of this document). This reference discloses IL-12 binding to the IL-12 receptors on the surface of B cells and indicates that this binding results in enhancement of humoral immunity (TH2 response) by upregulation of antibody production by B cells. As described earlier, since antibodies are mediators of humoral immunity, the results disclosed in Metzger indicate that IL-12 enhances the activity of B cells. Therefore, the Patent Office's characterization of IL-12 as an effector of B cell depletion in incorrect. Rather, IL-12, acting directly on B cells, increases the

ability of B cells to contribute to a humoral immune response through increased antibody production.

Additionally, Appellant reiterates two arguments against IL-12 as an effector of B cell depletion that were made in a response entered October 25, 2004 (see page 15 of "Amendment A"). Trinchieri indicates that IL-4 is a major cytokine produced during a TH2, or humoral response (see Figure 2 of Trinchieri). Trinchieri states (page 337, column 1, lines 35-37) that the "effect of IL-4 is, however, dominant over that of IL-12." Since IL-4 would be present during a TH2 immune response (when B cells are present), its dominance over IL-12 would preclude any IL-12 activity of the type that would effect B cell depletion. This is supported by the further disclosure in Trinchieri that "once a TH1 or TH2 type of response is determined early during the immune response, it remains stable, unless major changes take place in the balance of cytokine production during the response" (Trinchieri, page 337, column 2, lines 42-48). Therefore, Trinchieri indicates that IL-12 is not an effector of B cell depletion.

The Patent Office, in the final Office Action mailed January 26, 2005, rejected this argument stating that the intended use of a claimed product carries no patentable weight (page 5). Appellant believes the Patent Office has missed the point here. The point is that IL-12 is not an effector of B cell depletion. Appellant's claims recite an immunotherapeutic composition for effecting B cell depletion. An immunotherapeutic composition that does not effect B cell depletion (i.e., IL-12) does not anticipate Appellant's claims. Appellant's argument has nothing to do with intended use of the claimed composition.

In reiterating a second argument, IL-12 should not be interpreted as an effector of B cell depletion, based on experimental evidence in the specification as filed (e.g., see specification page 40, lines 4-13, and Figure 4). In the described study, mice with tumors were treated with either anti-mouse IgM to effect B cell depletion, or were treated with anti-mouse IgM plus IL-12. The data in Figure 4 show that the treatment with IgM plus IL-12 (Figure 4, line 3) was significantly less effective in preventing recurrence of tumors than the treatment with IgM alone (Figure 4, line 2). If IL-12 was an effector of B cell depletion, it would be expected that animals treated with IgM plus IL-12 would have less tumor recurrence than animals treated with IgM alone. This is true because, as described above, an overactive humoral, or protumor immune response promotes tumor progression. Depletion of B cells that contribute to the humoral response, therefore, would have decreased tumor recurrence in this study.

In the final Office Action mailed January 26, 2005, the Patent Office rejected this argument (page 6), stating that the argument "appears to go more towards enablement of the claimed composition." The Patent Office also stated (page 6) "that Figure 4 and the relevant text at page 40 does not indicate what immunomodulator was actually used and there are many such immunomodulators disclosed at pages 11-12 of the specification."

Appellant is bewildered by these comments. First, Appellant's argument does not bear on enablement. Rather, Appellant's argument is based on a study described in the specification suggesting that IL-12 is not an effector of B cell depletion. Again, since Appellant's claims recite an immunotherapeutic composition for effecting B cell depletion, if IL-12 does not effect B cell depletion, Noguchi does not anticipate Appellant's claims. Second, in the paragraph beginning at the top of page 40, the specification clearly states that the "immunomodulator" is IL-12. The study, whose results are shown in Figure 4 of the specification, is exactly as has been described. There is no mystery.

Therefore, lacking disclosure of a composition comprising an immunotherapeutic composition for effecting B cell depletion and a tumor-associated antigen capable of inducing a cell mediated immune response, Noguchi, even as evidenced by Trinchieri, does not anticipate Appellant's claims 1 and 9. Claims 2, 7 and 8 depend from claim 1 and claim10 depends from claim 9. Claims 2, 7, 8 and 10, therefore, are also not anticipated by Noguchi as evidenced by Trinchieri.

B. Claims 1-5, 7-12, 69-73, 77, 78, 80-86, 90, 92-96, 100, 102-108, 112, 114 And 115 Are Patentable Over Apostolopoulos In View Of Tachibana, Trinchieri, Parkhouse And Wang

Claims 1-5, 7-12, 69-73, 77, 78, 80-86, 90, 92-96, 100, 102-108, 112, 114 and 115 are not obvious in view of the combination of Apostolopoulos, Tachibana, Trinchieri, Parkhouse and Wang under 35 U.S.C. § 103(a).

1. Brief Discussion of the Apostolopoulos, Tachibana, Trinchieri, Parkhouse and Wang References

The study described in Apostolopoulos discloses that administration of a tumor-associated antigen conjugated to a carbohydrate polymer (mannan) produced a cell

mediated immune response that is effective against tumors, in contrast to previous reports where administration of this tumor-associated antigen, conjugated to carriers, produced a humoral immune response and ineffective antitumor activity.

The study described in Tachibana discloses that induction of a tumor-specific humoral immune response in tumor-bearing mice, by administration of tumor-associated antigens in the context of tumor cell lines, caused enhancement of tumor growth. The study also showed that, when induction of humoral immunity was inhibited, through the use of the drug cyclophosphamide, that the enhanced tumor growth was not present.

The disclosure of Trinchieri was described earlier.

The study described in Parkhouse discloses that an anti-CD22 antibody, conjugated to ricin, depletes B cells.

The study described in Wang discloses solid phase implants for delivery of biological macromolecules.

2. Argument

Appellant's pending independent claims 1 and 9 recite a composition comprising an immunotherapeutic composition for effecting B cell depletion and a tumor-associated antigen. As stated by the Patent Office, these claims are being examined to the extent that the immunotherapeutic composition for effecting B cell depletion is a monoclonal antibody specific for CD22. Independent claims 69, 82, 92 and 104 recite a composition comprising a monoclonal antibody specific for CD22 for effecting B cell depletion and a tumor-associated antigen capable of inducing a cell mediated immune response.

Since no single reference discloses all the elements of the above claims, the Patent Office has rejected the claims as being obvious over a combination of art (i.e., Apostolopoulos, Tachibana, Trinchieri, Parkhouse and Wang) under 35 U.S.C. § 103(a). Under 35 U.S.C. §103(a), a *prima facie* case of obvious is established if: 1) the prior art reference (or references if combined) discloses or suggests all the claim limitations (MPEP § 2143.03); 2) there is some suggestion or motivation to modify the reference to supply the missing teaching (or to combine separate references) (*In re Fine*, 837 F.2d 1071, 1074, 5 USPQ2d 1596, 1598 (Fed Cir. 1988)), and; 3) there is a reasonable expectation of success when the reference is modified (or combined) (*Yamanouchi Pharm Co. v. Danbury Phamacal, Inc.*, 231 F.3d 1339, 1343, 56

USPQ2d 1641, 1644 (Fed Cir. 2000)). Respectfully, Appellant submits that the Examiner failed to establish a *prima facie* case of obviousness regarding this rejection, based on a lack of motivation to combine the references.

With regard to motivation to combine the references, Appellant notes that it is not enough that one may modify a reference in view of a second reference, but rather it is required that one reference suggests the modification of the second reference. The Patent Office cannot use Appellant's claims as a roadmap for choosing distinct elements from the prior art without also showing motivation to combine separate references. The Court of Appeals for the Federal Circuit has restated numerous times the requirement of a teaching or suggestion to combine elements in the prior art:

> When it is necessary to select elements of various teachings in order to form the claimed invention, we ascertain whether there is any suggestion or motivation in the prior art to make the selection made by the applicant. ... "'Obviousness can not be established by combining the teachings of the prior art to produce the claimed invention, absent some teaching, suggestion, or incentive supporting the combination.'" ...
>
> ... It is impermissible ... simply to engage in hindsight reconstruction of the claimed invention, using the applicant's structure as a template and selecting elements from references to fill the gaps. ... **The references themselves must provide some teaching whereby the applicant's combination would have been obvious.**

(*In re Gorman*, 933 F.2d 982, 987, 18 U.S.P.Q.2d 1885, 1888 (Fed. Cir. 1991) (citations omitted) (emphasis added)).

Appellant notes that, of the Apostolopoulos, Tachibana, Trinchieri, Parkhouse and Wang references, the only reference that discloses an immunotherapeutic composition for effecting B cell depletion is Parkhouse, where an anti-CD22 antibody that is conjugated to the cellular toxin, ricin, is disclosed. There is simply no motivation within Parkhouse, or in the other references, to make the combination proposed by the Patent Office. Appellant's claims recite an anti-CD22 antibody to effect B cell depletion, while Parkhouse discloses an anti-CD22 antibody conjugated to the cellular toxin, ricin. Given the anti-CD22 antibody-ricin conjugate of Parkhouse, at best, it might have been obvious to try anti-CD22 antibody alone to see if B cells could be depleted. However, "obvious to try" is not sufficient to

provide a motivation to combine references under 35 U.S.C. § 103(a) (*In re O'Farrell*, 853 F.2d 894, 903, 7 USPQ2d 1673, 1681 (Fed. Cir. 1988)). Therefore, Appellant does not believe that there is motivation within Parkhouse to combine this reference with a reference disclosing a tumor-associated antigen, as would be required to render the pending claims obvious. Additionally, Appellant does not believe there is motivation to combine with Parkhouse in the other references cited by the Patent Office. None of those references (Apostolopoulos, Tachibana, Trinchieri and Wang) teach or suggest specifically depleting B cells and certainly do not teach or suggest depleting B cells with an anti-CD22 antibody.

Additionally, there must be a reasonable expectation that combining of references or modifying a reference would produce a successful result (*Yamanouchi Pharm Co. v. Danbury Phamacal, Inc.*, 231 F.3d 1339, 1343, 56 USPQ2d 1641, 1644 (Fed Cir. 2000)). Appellant's claims recite an anti-CD22 antibody to effect B cell depletion. However, Parkhouse discloses an anti-CD22 antibody conjugated to ricin. In order for Parkhouse to supply the teaching of depleting B cells, there would have to be a reasonable expectation that modifying the anti-CD22-ricin conjugate to anti-CD22 without the conjugated ricin would be able to effect B cell depletion. Given the high toxicity of ricin (see Lord, J. M., et al. 1994. Ricin: structure, mode of action, and some current applications. FASEB J. 8:201-208; a copy of this reference is in the Evidence Appendix beginning on page 25 of this document), there is not a reasonable expectation that anti-CD22 antibody alone, without ricin, would be successful in effecting B cell depletion. Therefore, Appellant does not believe that there is a reasonable expectation that modification of the disclosure in Parkhouse, to anti-CD22 antibody alone, would provide the required reasonable expectation of success.

In the final Office Action mailed January 26, 2005, the Patent Office stated (pages 9 and 10) that the motivation to combine the cited references comes from Apostolopoulos. Apostolopoulos discloses administration of a particular, mannan-conjugated tumor-associated antigen to produce a cell mediated immune response that is effective against tumors, where previous tumor-associated antigens, conjugated to carriers, produced a humoral immune response and inefficient antitumor activity. Although Apostolopoulos discloses tumor-specific antigens, Apostolopoulos does not disclose or suggest depleting B cells. Apostolopoulos merely discloses that conjugation of tumor-specific antigens with certain carriers may facilitate a tumor-specific antigen in producing a cell mediated immune response rather than a humoral

immune response. There is no teaching or suggestion within Apostolopoulos that humoral immunity could or should be suppressed. There is no motivation to attempt to deplete B cells. There is no motivation to use an anti-CD22 antibody to deplete B cells.

The Patent Office also stated in the final Office Action (page 9) that motivation to combine the references also comes from Tachibana, which states "immune complexes interfere with cell-mediated immunity to cause enhancement of tumor growth" and "the enhancement of tumor growth was caused by acceleration of humoral response existing beforehand in the tumor-bearing state." Disclosing that humoral immune responses inhibit cell mediated antitumor activity is not the same as disclosing or suggesting depletion of B cells or depletion of B cells with anti-CD22 antibody to eliminate a humoral immune response. Again, the only reference cited by the Patent Office that discloses an immunotherapeutic composition for effecting B cell depletion is Parkhouse. One would not be motivated to combine Tachibana with Parkhouse to come up with a composition containing an anti-CD22 antibody that alone is sufficient to deplete B cells. The anti-CD22 antibody disclosed in Parkhouse is conjugated to ricin. While it might be "obvious to try" anti-CD22 antibody without ricin to deplete B cells based on Parkhouse, that something is obvious to try is not sufficient motivation to combine or modify a reference (*In re O'Farrell*).

C. Claims 70, 71, 73, 78, 82-84, 86, 89, 90, 93, 94, 96, 100, 105, 106, 108 And 112 Meet The Written Description Requirement

Claims 70, 71, 73, 78, 82-84, 86, 89, 90, 93, 94, 96, 100, 105, 106, 108 and 112 meet the written description requirement 35 U.S.C. § 112, first paragraph.

1. Argument

In the final Office Action mailed January 26, 2005, the Patent Office stated (pages 11-14) that certain claim elements were not described in the specification in a manner that reasonable conveys to one skilled in the art that the inventor was in full possession of the invention at the time the application was filed. More specifically, the Patent Office referenced the Written Description Guidelines for examination of patent applications (page 12) which states:

> the written description requirement for a claimed genus may be satisfied through sufficient description of a representative number of species, by actual reduction to practice, or by disclosure of relevant, identifying characteristics, i.e., structure or

other physical characteristics and/or other chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics, sufficient to show applicant was in possession of the claimed genus.

(Federal Register 66:1099-1111; MPEP § 2164 (emphasis added)).

In particular, the Patent Office stated (final Office Action, page 12) that the words "immunomodulator" (claims 2, 10, 70, 72, 73, 83-86, 93-96 and 105-108) as used in the phrase "immunomodulator for inducing a cell mediated immune response comprising a Th1" and the phrase "anti-B cell agent" (claims 4, 11, 78, 90 and 100) as used in the phrase "an anti-B cell agent for effecting B cell depletion" do not meet the written description requirement. The Patent Office asserts that there is not sufficient description in the specification of these terms (e.g., relevant identifying characteristics, such as physical and/or chemical properties and structure) to demonstrate full possession of the invention at the time the application was filed.

This assessment is incorrect. Appellant below, cites portions of the specification as filed with respect to both "immunomodulator" and "anti-B cell agent." Based on these passages from the specification, it is clear that these descriptions meet the written description requirements, as set forth in the passage from the Written Description Guidelines, cited above.

As to the term "immunomodulator," attention is directed to the paragraph beginning on page 11 of the application. This paragraph is reproduced in its entirety below, with underlined portions directed to representative species of immunomodulators:

> The term "immunomodulator" is used herein, for purposes of the specification and claims, to mean one or more compositions that, when administered to an individual in an effective amount, induces a cell mediated immune response comprising a TH1 response, and more preferably, may also induce a cytotoxic CD8+ T cell response. As known to those skilled in the art, a composition that may induce a cell mediated immune response comprising a TH1 response may include, but is not limited to, IL-12, IL-12 and melatonin, flavone acetic acid (flavonoid, 2-heteroaryl flavonoid derivatives, flavone-8-acetic acid), QS-21(a purified form of saponin, at a high dose) and monophosphoryl lipid A, N-acetylcysteine, SAF-1 (Syntex adjuvant formulation-1), AS101 (ammonium trichloro (dioxoethylene-O,O') tellurate), lentinan (a fungal branched 1.fwdarw.3-(beta)-D-glycan), TraT protein ("ISCAR" or immunostimulatory carrier- an integral membrane protein of E. coli), Viscum album extract (commercially available extract of mistletoe), Z-100 (a lipid arabinomannan-containing extract of M. tuberculosis), OK-432 (Picibanil, inactivated and heat treated S. pyogenes Su strain), immunostimulatory DNA sequences, and the like. For example, IL-12 has

been shown to be a potent inducer of naive CD4+ cells towards a cell mediated immune response comprising a TH1 response. IL-12 may be administered to a human individual as a cytokine in solution (e.g., rlL-12 in a dose ranging from about 10 ng/kg to about 300 ng/kg, twice weekly, subcutaneously or intratumoral), or in the form of dendritic cells or fibroblasts genetically engineered to express human IL-12 (see, e.g., Lotze et al., 1997, Cancer J. Sci. Am. 3/S (S109-S114), herein incorporated by reference). In another example, short bacterial immunostimulatory DNA sequences containing unmethylated CpG motifs have been shown to be able to stimulate a TH1 response (e.g., by inducing IL-12 production), and hence stimulate a cell-mediated immune response (Roman et al., 1997, Nat. Med. 3:849-854; Lipford et al., 1997, Eur. J. Immunol. 27: 3420-3426, herein incorporated by reference). An amount of an immunomodulator effective to induce a cell mediated immune response comprising a TH1 response will vary depending on such factors as the mode of administration, individual's age, weight, general medical condition, and immune status. For purposes of illustration, but not limitation, lentinan has been administered intravenously (e.g., 2 mg, 3 times per week), melatonin has been administered orally (e.g., 20 mg/day in the evening), Viscum album has been administered subcutaneously (e.g., 2-3 times per week, ranging from 0.1 to 30 mg), AS101 has been administered by intravenous drip (e.g., in a range of from about 3 mg/m.sup.2 to about 12 mg/m.sup.2), and QS-21 has been administered subcutaneously (e.g., in a range of from about 100 .mu.g to about 200 μg). A preferred immunomodulator may be used in the present invention to the exclusion of an immunomodulator other than the preferred immunomodulator.

This paragraph provides more than sufficient description of the term "immunomodulator" to meet the written description requirement. At the very least, the paragraph provides sufficient description of a representative number of species of immunomodulators to demonstrate full possession of the invention.

As to the term "anti-B cell agent," attention is directed to the paragraph beginning at the bottom of page 8 of the application. Portions of this paragraph are reproduced below, with underlined portions directed to representative species of immunomodulators:

In another embodiment the affinity ligand, which comprises the immunotherapeutic composition, may further comprise at least one anti-B cell agent. The "anti-B cell agent" comprises a cytolytic agent (e.g., the agent itself or a vector that is introduced into B cells and therein the vector encodes a cytolytic agent). The anti-B cell agent may be coupled to the affinity ligand using methods known in the art for coupling affinity ligands to other molecules (See, for example, conjugates as reviewed by Ghetie et al., 1994, Pharmacol. Ther. 63:209-34; U.S. Pat. No. 5,789,554, the disclosure of which is herein incorporated by reference). Often such methods utilize one of several available heterobifunctional reagents used for coupling or linking molecules. The affinity ligand serves to selectively bind the B cells, thereby bringing the anti-B cell agent in contact with

or in functional proximity of B cells. A cytolytic agent is an agent that, by interacting directly with such B cells, causes B cell cytotoxicity. Such cytolytic agents may include, but are not limited to, a therapeutically effective amount of toxins; drugs; enzymes; cytokines; radionuclides; photodynamic agents; and molecules which induce apoptosis (e.g., Fas ligand; a Fas ligand expressing vector has been described in more detail in by the present inventor in Gene Therapy 8:209-214, 2001, the disclosure of which is herein incorporated by reference). Toxins may include a cytolytically effective amount of ricin A chain, mutant Pseudomonas exotoxins, diphtheria toxoid, streptonigrin, boamycin, saporin, gelonin, pokeweed antiviral protein, or the like. Drugs may include an effective amount of cytotoxic drug including, but not limited to, fludarabine, chlorambucil, daunorubicin, doxorubicin (e.g., in liposomes), cisplatin, bleomycin, melphalan, mitomycin-C, and methotrexate. A preferred cytotoxic drug may be used as an anti-B cell agent in the present invention to the exclusion of a cytotoxic drug other than the preferred cytotoxic drug. Due to the sensitivity of B cells to radiation, a radionuclide may include, but is not limited to, a radiometal such as yttrium which emits a high energy beta particle, and I^{125} that emits Auger electrons, that may be absorbed by adjacent B cells. A photodynamic agent may include a cytolytically effective amount of a porphyrin or a porphryin derivative as known in the art. A preferred anti-B cell agent may be used in the present invention to the exclusion of an anti-B cell agent other than the preferred anti-B cell agent.

This passage provides more than sufficient description of the term "anti-B cell agent" to meet the written description requirement. At the very least, the paragraph provides sufficient description of a representative number of species of anti-B cell agents to demonstrate full possession of the invention.

VIII. CONCLUSION

Appellant believes that the remarks presented above resolve all outstanding issues concerning the above-referenced application. Accordingly, Appellant believes that the appealed claims are allowable, and urges reversal of the final rejections and allowance of the appealed claims.

While no fees are believed due, the Commissioner is hereby authorized to charge any additional fees, or credit any overpayment to Deposit Account No. 02-2051, referencing Attorney Docket No. 26983-98.

Respectfully submitted,

Dated: 6/22/2005

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IX. CLAIMS APPENDIX

1. (previously presented) A composition for treating a TH2 response and for inducing a cell mediated immune response comprising a TH1 response in an individual having a TH2/TH1 imbalance associated with a pro-tumor immune response, the composition comprising: an immunotherapeutic composition for effecting B cell depletion; and tumor-associated antigen capable of inducing a cell mediated immune response comprising a TH1 response.

2. (previously presented) The composition according to claim 1, further comprising a component selected from the group consisting of an immunomodulator for inducing a cell mediated immune response comprising a TH1 response, a pharmaceutically acceptable carrier, and a combination thereof.

3. (previously presented) The composition according to claim 1, wherein the immunotherapeutic composition is contained in a solid phase implant for delivery of the immunotherapeutic composition.

4. (previously presented) The composition according to claim 1, wherein the immunotherapeutic composition further comprises an anti-B cell agent.

5. (previously presented) The composition according to claim 1, wherein the immunotherapeutic composition comprises an affinity ligand having binding specificity for a determinant selected from the group consisting of CD19, CD20, CD21, CD22 (also known as LL2), CDIM, and Lym-1.

6. (canceled)

7. (previously presented) The composition according to claim 1, wherein the TH2/TH1 imbalance is mediated by a disease process comprising a pro-tumor immune response.

8. (previously presented) The composition according to claim 1, wherein the TH2/TH1 imbalance is mediated by a disease process comprising a pro-tumor immune response and solid nonlymphoid tumor.

9. (previously presented) A composition useful for the treatment of solid nonlymphoid tumor in an individual, the composition comprising: an immunotherapeutic composition for effecting B cell depletion; and tumor-associated antigen capable of inducing a cell mediated immune response comprising a TH1 response;

wherein the composition is in an amount effective to overcome a TH2/TH1 imbalance, the TH2/TH1 imbalance associated with a pro-tumor immune response, or a combination of the solid nonlymphoid tumor and a pro-tumor immune response.

10. (previously presented) The composition according to claim 9, further comprising a component selected from the group consisting of an immunomodulator for inducing a cell mediated immune response comprising a TH1 response, a pharmaceutically acceptable carrier, and a combination thereof.

11. (previously presented) The composition according to claim 9, wherein the immunotherapeutic composition further comprises an anti-B cell agent.

12. (previously presented) The composition according to claim 9, wherein the immunotherapeutic composition comprises an affinity ligand having binding specificity for a determinant selected from the group consisting of CD19, CD20, CD21, CD22 (also known as LL2), CDIM, and Lym-1.

13-68. (canceled)

69. (previously presented) A composition comprising:

(a) an immunotherapeutic composition comprising a monoclonal antibody having binding specificity for CD22 for effecting B cell depletion; and

(b) tumor-associated antigen capable of inducing a cell mediated immune response comprising a TH1 response;

wherein the composition is in an amount effective for suppressing a TH2 response, and for inducing a cell mediated immune response comprising a TH1 response, in an individual having a TH2/TH1 imbalance associated with a pro-tumor immune response.

70. (previously presented) The composition according to claim 69, further comprising a component selected from the group consisting of an immunomodulator for inducing a cell mediated immune response comprising a TH1 response, a pharmaceutically acceptable carrier, and a combination thereof.

71. (previously presented) The composition according to claim 69, further comprising an immunomodulator for inducing a cell mediated immune response comprising a TH1 response.

72. (previously presented) The composition according to claim 69, wherein the immunotherapeutic composition further comprises a pharmaceutically acceptable carrier, and the tumor-associated antigen further comprises a pharmaceutically acceptable carrier.

73. (previously presented) The composition according to claim 70, wherein the component comprises an immunomodulator and a pharmaceutically acceptable carrier.

74-76. (canceled)

77. (previously presented) The composition according to claim 74, wherein the immunotherapeutic composition is contained in a solid phase implant for delivery of the immunotherapeutic composition.

78. (previously presented) The composition according to claim 69, wherein the immunotherapeutic composition further comprises an anti-B cell agent.

79. (canceled)

80. (previously presented) The composition according to claim 69, wherein the TH2/TH1 imbalance is mediated by a disease process comprising a pro-tumor immune response.

81. (previously presented) The composition according to claim 69, wherein the TH2/TH1 imbalance is mediated by a disease process comprising a pro-tumor immune response and solid nonlymphoid tumor.

82. (previously presented) A composition comprising:

(a) an immunotherapeutic composition comprising a monoclonal antibody having binding specificity for CD22 for effecting B cell depletion; and

(b) tumor-associated antigen capable of inducing a cell mediated immune response comprising a TH1 response;

wherein the composition is in an effective amount for the treatment, or inhibition of development, of solid nonlymphoid tumor in an individual having a pro-tumor immune response.

83. (previously presented) The composition according to claim 82, further comprising a component selected from the group consisting of an immunomodulator for inducing a cell mediated immune response comprising a TH1 response, a pharmaceutically acceptable carrier, and a combination thereof.

84. (previously presented) The composition according to claim 82, further comprising an immunomodulator for inducing a cell mediated immune response comprising a TH1 response.

85. (previously presented) The composition according to claim 82, wherein the immunotherapeutic composition further comprises a pharmaceutically acceptable carrier, and the tumor-associated antigen further comprises a pharmaceutically acceptable carrier.

86. (previously presented) The composition according to claim 83, wherein the component comprises an immunomodulator and a pharmaceutically acceptable carrier.

87-89. (canceled)

90. (previously presented) The composition according to claim 82, wherein the immunotherapeutic composition further comprises an anti-B cell agent.

91. (canceled)

92. (previously presented) A composition comprising:

(a) an immunotherapeutic composition comprising a monoclonal antibody having binding specificity for CD22, for effecting B cell depletion in suppressing a TH2 response associated with a pro-tumor immune response or a combination of a pro-tumor immune response and solid nonlymphoid tumor; and

b) tumor-associated antigen capable of inducing a cell mediated immune response comprising a TH1 response.

93. (previously presented) The composition according to claim 92, further comprising a component selected from the group consisting of an immunomodulator for inducing a cell mediated immune response comprising a TH1 response, a pharmaceutically acceptable carrier, and a combination thereof.

94. (previously presented) The composition according to claim 93, further comprising an immunomodulator for inducing a cell mediated immune response comprising a TH1 response.

95. (previously presented) The composition according to claim 92, wherein the immunotherapeutic composition further comprises a pharmaceutically acceptable carrier, and the tumor-associated antigen further comprises a pharmaceutically acceptable carrier.

96. (previously presented) The composition according to claim 93, wherein the component comprises an immunomodulator and a pharmaceutically acceptable carrier.

97-99. (canceled)

100. (previously presented) The composition according to claim 92, wherein the immunotherapeutic composition further comprises an anti-B cell agent.

101. (canceled)

102. (previously presented) The composition according to claim 92, wherein the TH2/TH1 imbalance is mediated by a disease process comprising a pro-tumor immune response.

103. (previously presented) The composition according to claim 92, wherein the TH2/TH1 imbalance is mediated by a disease process comprising a pro-tumor immune response and solid nonlymphoid tumor.

104. (previously presented) A composition comprising:

(a) an immunotherapeutic composition comprising a monoclonal antibody having binding specificity for CD22 for effecting B cell depletion in suppressing a TH2 response; and

(b) tumor-associated antigen capable of inducing a cell mediated immune response comprising a TH1 response;

wherein the composition is in an amount effective to overcome a TH2/TH1 imbalance associated with a pro-tumor immune response, or a combination of solid nonlymphoid tumor and a pro-tumor immune response.

105. (previously presented) The composition according to claim 104, further comprising a component selected from the group consisting of an immunomodulator for inducing a cell mediated immune response comprising a TH1 response, a pharmaceutically acceptable carrier, and a combination thereof.

106. (previously presented) The composition according to claim 104, further comprising an immunomodulator for inducing a cell mediated immune response comprising a TH1 response.

107. (previously presented) The composition according to claim 104, wherein the immunotherapeutic composition further comprises a pharmaceutically acceptable carrier, and the tumor-associated antigen further comprises a pharmaceutically acceptable carrier.

108. (previously presented) The composition according to claim 105, wherein the component comprises an immunomodulator and a pharmaceutically acceptable carrier.

109-111. (canceled)

112. (previously presented) The composition according to claim 104, wherein the immunotherapeutic composition further comprises an anti-B cell agent.

113. (canceled)

114. (previously presented) The composition according to claim 104, wherein the TH2/TH1 imbalance is mediated by a disease process comprising a pro-tumor immune response.

115. (previously presented) The composition according to claim 104, wherein the TH2/TH1 imbalance is mediated by a disease process comprising a pro-tumor immune response and solid nonlymphoid tumor.

X. EVIDENCE APPENDIX

1. Metzger, D. W., et al. 1996. Enhancement of humoral immunity by interleukin-12. Ann. NY Acad. Sci. 795:100-115. (An abstract of this reference is attached at the end of this document).

2. Lord, J. M., et al. 1994. Ricin: structure, mode of action, and some current applications. FASEB J. 8:201-208. (The complete reference is attached at the end of this document).

1: Ann N Y Acad Sci. 1996 Oct 31;795:100-15.

Enhancement of humoral immunity by interleukin-12.

Metzger DW, Buchanan JM, Collins JT, Lester TL, Murray KS, Van Cleave VH, Vogel LA, Dunnick WA.

Department of Microbiology, Medical College of Ohio, Toledo 43699-0008, USA.

We have found that IL-12 treatment of mice leads to long-lasting enhancement in production of most antibody isotypes in conventional B-cell responses. Initial recruitment of new B-cell clones into the response is mediated by IFN-gamma, but subsequent enhancement of Ig secretion appears to be IFN-gamma-independent. We have further found that activated B cells can directly bind IL-12. Taken together, our results suggest a two-step model for the role of IL-12 in enhancement of humoral immunity. Initially, IL-12 induces production of IFN-gamma from Th1 and NK cells. Enough cytokine can be produced from either cell type to then mediate gamma 2a heavy chain isotype switching as well as temporary suppression of IgG1 production. IL-12 further stimulates post-switched cells, including cells producing IgG1, to secrete greatly increased amounts of antibody. This step is not mediated by IFN-gamma but might be due to direct IL-12 binding to activated B lymphocytes. Depletion of B1 cells by IL-12 may further enhance antibody responsiveness since B1 cells are known to competitively inhibit Ig secretion by conventional B cells. The end result is that IL-12 causes a generalized upregulation in production of all antibodies and therefore acts as a strong adjuvant for humoral as well as cellular immunity.

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Ricin: structure, mode of action, and some current applications

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ABSTRACT **Ricin is an abundant protein component of *Ricinus communis* seeds (castor beans) that is exquisitely toxic to mammalian cells. It consists of an enzymic polypeptide that catalyzes the N-glycosidic cleavage of a specific adenine residue from 28S ribosomal RNA, joined by a single disulfide bond to a galactose (cell)-binding lectin. The enzymatic activity renders ribosomes containing depurinated 28S RNA incapable of protein synthesis. The bipartite molecular structure of ricin allows it to bind to the mammalian cell surface, enter via endocytic uptake, and deliver the catalytically active polypeptide into the cell cytosol where it irreversibly inhibits protein synthesis causing cell death. Because of its cytotoxic potency, modified ricin is being used for the selective killing of unwanted cells and for the toxigenic ablation of cell lineages in transgenic organisms.—Lord, J. M., Roberts, L. M., Robertus, J. D. Ricin: structure, mode of action, and some current applications. *FASEB J.* 8: 201-208; 1994.**

Key Words: ricin • ribosome inactivating protein • lectin • depurination • endocytosis

RICIN IS A POTENTLY TOXIC PROTEIN found in castor oil plant (*Ricinus communis*) seeds. Many plants contain poisonous substances that have attracted the attention both of physicians, who have frequently found the sparing use of plant toxins to be therapeutically beneficial, and criminals, who have tended to be more liberal in the doses they use. Ricin fits readily into this classification; its anti-cancer potential, particularly as a component of immunotoxins, has attracted considerable attention recently, as did its more sinister reputed use in the assassination of the Bulgarian journalist Georgi Markov in London in 1978 (1).

The toxicity of *Ricinus* seeds has been recognized since ancient times. More than a century ago, Stillmark isolated a toxic protein from the seeds, which he termed ricin (2). Toxicity was believed to result from the observed ability of the ricin preparation to agglutinate red blood cells. More recent studies established that Stillmark's ricin preparations were a mixture of a potent cytotoxin (ricin) and a hemagglutinin (*Ricinus communis* agglutinin). Several workers, including Alexander Pihl, Sjur Olsnes, and their colleagues working in Norway, showed that the toxicity of ricin is due to its catalytic action on eukaryotic ribosomes. This work culminated in 1987 when Yaeta Endo and colleagues working in Japan identified the mechanism of action of ricin on 28S ribosomal RNA.

Since the isolation of ricin, many structurally and functionally related proteins have been characterized from a wide variety of higher plants. All these proteins are enzymes that specifically and irreversibly inactivate eukaryotic ribosomes such that the latter can no longer participate in protein synthesis. Collectively known as ribosome-inactivating proteins (RIPs),[1] these proteins usually occur as monomers of approximately 30 kDa (called type I RIPs) and are frequently, but not always, N-glycosylated (3). In spite of their ribosome-inactivating activity, type 1 RIPs are not cytotoxic because they appear to have no means of entering eukaryotic cells in order to reach their ribosomal substrates. Indeed, certain plant tissues that are rich in type I RIPs, such as wheat germ or barley grain, are widely consumed by humans and animals. In certain plant tissues, however, the RIP is covalently joined through a disulfide bond to a second polypeptide, which in all cases described to date is a galactose-binding lectin whose molecular mass is also around 30 kDa. These heterodimeric toxins (type II RIPs) bind to eukaryotic cells by interacting with cell-surface galactosides, and after subsequently entering the cytosol are able to promote cell death by inhibiting protein synthesis. This group, including the toxic proteins abrin and modeccin, contains some of the most potent cytotoxins in nature. The most extensively characterized member of the group, ricin, is described in some detail here.

OCCURRENCE, STRUCTURE, AND BIOGENESIS

There are several isoforms of ricin including ricin D, ricin E, and the closely related lectin *Ricinus communis* agglutinin (RCA). Together they account for more than 5% of the total protein present in mature *Ricinus* seeds. Ricin is a heterodimeric type II RIP composed of a ribosome-inactivating enzyme (32 kDa, designated the A chain or RTA) linked to a galactose/N-acetylgalactosamine-binding lectin (34 kDa, the B chain or RTB) by a single disulfide bond. RCA in contrast is tetrameric, composed of two ricin-like heterodimers, each of which contains an A chain (32 kDa) and a galactose-binding B chain (36 kDa). In addition to their structural differences, these two proteins also differ in their biological properties. Whereas ricin is a potent cytotoxin but a weak hemagglutinin, RCA is only weakly toxic to intact cells but is a strong hemagglutinin. However, although RCA is only weakly toxic to intact cells in vivo, its isolated A chain is an RIP of comparable activity to ricin A chain in terms of its ability to modify ribosomes in vitro (4). The sugar binding specificities are also similar but not identical. Overall, however, ricin and RCA are closely related proteins and antisera raised against individual ricin A or B chains cross-

[1]Abbreviations: RiP, ribosome-inactivating protein; RTA, ricin toxin A chain; RTB, ricin toxin B chain; RCA, *Ricinus communis* agglutinin; TGN, trans Golgi network; ER, endoplasmic reticulum; BFA, brefeldin A; IT, immunotoxin; GVHD, graft-vs.-host disease; UAS, upstream activation sequence; FMP, formycin monophosphate; ts, temperature-sensitive; cs, cold-sensitive;

0892-6638/94/0008-0201/$01.50.

react strongly with the corresponding RCA subunits and vice versa. Indeed, the B subunit of ricin E appears to be a hybrid molecule in which the NH_2-terminal half of the protein closely resembles the B chain of ricin D in primary sequence whereas the COOH-terminal half clearly resembles the RCA B chain (5). The complete primary sequences of the ricin and RCA subunits have been determined chemically or deduced from the nucleotide sequence of cloned cDNAs and genes. The A chains of ricin D and RCA differ in 18 of their 267 residues and are thus 93% homologous at the amino acid level, whereas the B chains differ in 41 of 262 residues giving 84% homology (6).

Ricin and RCA are encoded by a small multigene family composed of approximately eight members, some of which are nonfunctional (7). Expression of the genes is tissue-specific and developmentally regulated. Ricin and its homologues are synthesized in the endosperm cells of maturing *Ricinus* seeds where the RIPs are targeted to an organelle called the protein body (analogous to a vacuolar compartment) for storage in the mature seed. When seeds subsequently germinate, the toxins are rapidly destroyed by hydrolysis over the first few days of postgerminate growth.

The ricin genes encode a preproprotein containing both the A and the B chain sequences. Preproricin consists of 576 amino acid residues, the first 35 of which include, but do not entirely consist of, an NH_2-terminal signal sequence, followed by the mature A chain sequence (267 residues) joined by a 12-residue linker peptide to the mature B chain sequence (262 residues). During ricin biosynthesis a series of cotranslational and post-translational processing steps have been elucidated.

Cotranslational modifications

During preproricin biosynthesis, the NH_2-terminal signal sequence directs the transport of the nascent polypeptide across the ER membrane into the ER lumen. Translocation across the ER membrane is accompanied by three major modifications (8). First, the NH_2-terminal signal peptide is cleaved by ER luminal signal peptidase. Although the precise cleavage site has not been determined by direct microsequencing, a computer prediction based on all known signal peptidase cleavage sites identified cleavage after Ser 22 in the 35-residue preproricin leader sequence as the most likely signal peptidase target site. The resulting proricin therefore still possesses a short amino acid sequence at the new NH_2 terminus before the mature A chain sequence begins. It has been speculated that this propeptide sequence might be a protein body targeting sequence, but direct evidence for this is lacking. Second, proricin is N-glycosylated as it enters the ER lumen. Proricin contains four N-glycosylation sites, two within the A chain sequence and two within the B chain. Third, protein disulfide isomerase catalyzes the formation of five disulfide bonds within the folding proricin. In mature ricin the RTB contains four intrachain disulfide bonds and is joined to RTA by another disulfide bond. These five disulfide bonds are introduced into proricin during or immediately after its synthesis: the bond destined to become the interchain disulfide joining RTA and RTB of mature ricin is formed between cysteine residues close to the COOH terminus of RTA (Cys 259) and the NH_2 terminus of RTB (Cys 4) in the precursor.

Post-translational modifications

Core-glycosylated, disulfide-bonded proricin is transported by vesicular flow from the ER lumen via the Golgi complex to the protein bodies where native heterodimeric ricin is proteolytically generated and stored (9). Transport through the Golgi complex is accompanied by the enzymatic modification of the oligosaccharide side chains of proricin. Although details of the sugar modifications are not known, they presumably involve typical Golgi oligosaccharide trimming and monosaccharide additions to the side chains. The latter include the addition of fucose and xylose to oligosaccharides on RTA. The sugar modifications confer partial endo-N-acetyl glucosamindase H resistance on the proricin sugar side chains (10). Finally, Golgi-derived vesicles carrying proricin fuse with the vacuolar membrane and discharge their contents into the protein body matrix. The targeting signal and sorting receptor required for protein body delivery have not been identified. Within the protein body matrix proricin is processed by at least one endopeptidase, which liberates RTA and RTB, still covalently joined by a disulfide bond because cleavage occurs within a disulfide loop connecting the A and B chain sequences. The endopeptidase shows maximum activity at pH4-pH5, the pH of the protein body matrix (11). This proteolytic processing step involves the removal of a 12-residue peptide that links the mature A and B chain sequences. The residual amino acid extension present at the NH_2 terminus of RTA after signal peptide cleavage is also believed to be excised at this time. The endoprotease responsible for the NH_2-terminal trimming and cleavage at the COOH-terminal end of the internal linker cleaves on the carboxyl side of appropriate asparagine residues, a step commonly involved in processing plant proproteins. Recently, a plant endoprotease with such specificity has been purified and characterized (12).

Significance of the post-translational modifications

Ricinus endosperm ribosomes are sensitive to the catalytic action of RTA, although plant ribosomes generally are significantly less sensitive to RIPs than their mammalian counterparts. However, in contrast to free RTA, the A chain portion of proricin is devoid of catalytic activity. Proricin is therefore transported from the ER to the protein bodies in a catalytically inactive form. Together with efficient targeting to the ER lumen during synthesis, this feature ensures that *Ricinus* ribosomes avoid the possibility of inactivation, even if some proricin were accidentally miscompartmentalized into the cytosol during its biosynthesis or intracellular transport.

CLONING AND EXPRESSION

Ricin was the first plant RIP to be cloned, and in 1985 both cDNA and genomic clones encoding preproricin were isolated, as was a cDNA clone encoding preproRCA (6, 13, 14). The DNA sequence of the genomic ricin clone showed that the gene did not contain introns. Analysis of the 5' untranslated and flanking regions of this gene revealed putative TATA and CAAT/AGGA consensus sequences. Another element associated with genes that are specifically expressed in plant seeds was also identified. Two separate motifs resembling the CATGCATG RY repeat previously noted in seed-specific genes were found, 113 bp and 143 bp upstream from the putative transcription start site. This motif may confer tissue and/or developmental specificity of expression in the ricin gene (7).

For expression in heterologous systems, DNA encoding the individual A or B chains, rather than the preproricin precursor, has usually been utilized. Two general approaches have been employed. Although RTA is a potent inhibitor of

80S eukaryotic ribosomes, it is without effect on 70S prokaryotic ribosomes, although this is not the case for many type I RIPs. Because of this, RTA can be expressed cytoplasmically in *Escherichia coli* as a nonglycosylated soluble product with full biological activity (15). When optimized, this system can routinely produce 1500 mg of active RTA per liter of culture (D. Thatcher, Zeneca Pharmaceuticals, personal communication). Purified recombinant RTA readily reassociates with RTB to produce a molecule whose cytotoxic potency is indistinguishable from the native holotoxin. RTB requires the correct formation of the four intrachain disulfide bonds for galactose-binding activity, and in this case N-glycosylation also confers stability on the molecule. Both of these modifications have been achieved by expressing RTB in eukaryotic systems, using an NH_2-terminal signal sequence to direct the recombinant product to the ER lumen of the heterologous host. RTB has been expressed by microinjecting in vitro-generated preRTB transcripts into *Xenopus* oocytes (16) or after introducing pre-RTB DNA, contained in appropriate vectors, into yeast or mammalian cells (17, 18). Alternatively, RTB can be expressed in *E. coli*. Provided an appropriate bacterial signal sequence is used to direct the product to the periplasmic space, RTB produced in *E. coli* is initially biologically active, but due to the lack of glycosylation, it is relatively unstable (19). The absence of glycosylation appears to be less crucial for stability if individual RTB subdomains are expressed independently (20).

Glycosylated proricin has also been expressed in *Xenopus* oocytes. The RTB component of the precursor has lectin activity but the RTA component is completely devoid of 28S rRNA N-glycosidase activity. The RTA component is correctly folded, however, as proteolytic cleavage of the precursor releases catalytically active RTA (21).

Figure 1. The backbone of ricin. The A chain is in the upper right as a braided ribbon. The adenine ring marks the active site cleft. The B chain is the solid ribbon. Lactose is shown binding at each end of the two domain peptide.

STRUCTURE OF RICIN

The three-dimensional structure of ricin has been solved by X-ray crystallography (22) and refined to 2.5 A resolution (23). This model allowed for an accurate and detailed description of both the A chain (24) and the B chain (25). In addition, the structure of recombinant RTA has been solved to 2.3 A resolution (26). **Figure 1** is a ribbon drawing of ricin showing the overall shape of both chains, their packing relations, and the location of key ligand binding sites. RTA is a 267-residue globular protein with a pronounced binding site cleft; RTB is a 262-residue elongated dumbbell with galactose binding sites at both ends.

RTA has eight alpha helices (A-H) and eight strands of beta sheet (a-h). The amino-terminal 117 residues, which is about 40% of the chain, form a compact folding unit. Beta strands 1-f form an extended mixed sheet, and together with helices A and B form the bottom of the molecule. The next 40% or so of the sequence is largely helical, containing helices C-G. These helices pack in a group resting on the sheet structure. Helix E, more than five turns in length, runs through the molecular center. It is largely nonpolar, but contains two crucial active site residues, Glu 177 and Arg 180, near its COOH terminus. These lie on consecutive helical turns and face into the solvent of the active site. The final 20% of the sequence of RTA folds as a compact unit but is anchored to helix A. One side of this unit helps form the active site cleft, and the other makes strong contacts with RTB in the heterodimer. The RTA fold is archetypal for the family of plant and bacterially derived RIPs.

The family of RIPs share only about eight invariant amino acids and most of these cluster in the active site. These include Tyr 80, Tyr 123, Glu 177, Arg 180, and Trp 211. RTA, like other RIPs, depurinates adenine in 28S rRNA. To investigate substrate binding, X-ray structures have been solved for complexes with the adenosine analog, formycin monophosphate (FMP) and the dinucleotide ApG (27). **Figure 2** shows an enlargement of the active site of RTA, together with bound FMP. The substrate purine ring is stacked between the rings of the two invariant tyrosines: Arg 180 bonds to N3 of the ring and Glu 177 is near the ribose. Trp 211 appears to make no specific contacts with the adenine but may be important for the active site conformation or may interact with a larger polynucleotide substrate.

As seen in Fig. 1, RTB is a two-domain structure. These two domains are homologous and arose by gene duplication; domain 1 is the amino-terminal half of the molecule and domain 2 the COOH-terminal half. What is not evident from the figure, but can be seen upon close analysis of the structure, is that each domain is formed from four subdomain units. Each contains a 17-residue linking peptide, lambda, and three homologous units, α, β, and γ, of a roughly 40-residue core peptide. These appear to have arisen from multiplication and fusion of DNA encoding an ancient galactose binding peptide, which could self assemble around a hydrophobic core into a trimeric structure. A detailed analysis of this structure and putative evolutionary scenario is given in ref 25. Of the six potential galactose binding sites, only 1α and 2γ still function. **Figure 3** shows an enlargement of the two binding sites based on the 2.5 refinement. The details differ from the model initially proposed for galactose

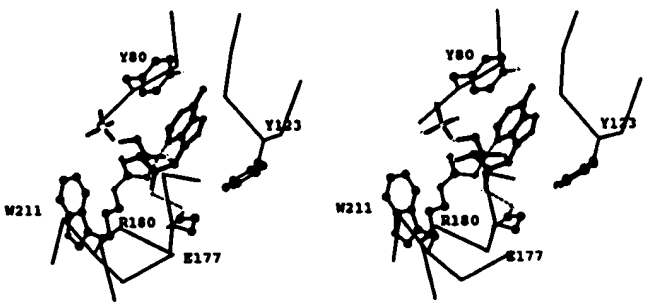

Figure 2. FMP bound in the active site of ricin A chain. The peptide chain is shown as line segments and key active site residues are labeled in the one letter code. Formycin monophosphate, an AMP analog, is shown in heavy bonds. Hydrogen bonds are indicated as dashed lines between polar atoms.

binding (22). The refined galactose binding pocket is quite shallow and interacts with only about half the sugar. The bottom of the pocket is formed by a three-residue kink in the peptide chain. The top is an aromatic side chain, Trp 37 and Tyr 248 in 1α and 2γ, respectively, which contacts the hydrophobic face of the sugar. Specific hydrogen bonds are made between the sugar and the two RTB sites that account for the epimeric specificity of binding. Asp 22 in site 1 and Asp 234 in site 2 make the primary interaction. They lie between the C3 and C4 hydroxyls and hydrogen bond to both. The crucial aspartates are locked in position by bonds from Gln 47 in site 1 and Gln 256 in site 2. The C3 hydroxyl of bound galactose also forms a strong hydrogen bond with Asn 46 in site 1 and Asn 255 in site 2. These interactions with galactose were thought to be the strongest, based on the unrefined model. Subsequent site-directed mutagenesis experiments confirmed that the Asn residues are important to galactose binding, but suggest that RTB can still bind to dense polysaccharides, like asialofetuin, even if they are disrupted (28, 29). Compared with sugar-sequestering proteins, the interactions between galactose and RTB are few in number (4 or 5 RTB amino acid residues are involved in contrast to, for example, 12 residues involved in the binding of arabinose to the *E. coli* L-arabinose binding protein (30)). This is consistent with the relatively low binding affinity, with K_a values in the range of 10^3 to 10^4.

MECHANISM OF ACTION OF RICIN A CHAIN

As described above, RTA is an N-glycosidase (31), depurinating adenine in a specific RNA sequence. The substrate conformation is thought to be important for recognition but is unknown for the actual ribosome substrate. The ricin target site in 28S rRNA is probably in a hairpin containing the tetranucleotide loop GAGA. The solution structure of such hairpins has been determined by NMR spectroscopy (32). The tetraloop is stabilized by an unusual G-A base pair between the first and last residues of the loop. It is known that tetraloop RNA can be attacked by RTA, and hypothetical models have been made for tetraloop binding to RTA based on the observed binding of mono- and dinucleotide (27). Although a complete understanding of ribosomal recognition remains to be elucidated, a reasonable mechanism can be proposed for depurination. Binding of the susceptible adenine is probably quite close to that of FMP and ApG described above. The catalytic importance of several active site residues identified by X-ray analysis has been investigated by site-directed mutagenesis in several laboratories (33-36). The main findings are these. Conversion of Arg 180 to Gln (R180Q) reduces activity about 2000-fold whereas conversion of Glu 177 to Gln reduces activity at least 200-fold. Kinetic analysis reveals that mutations at these two positions primarily affect k_{cat} with little effect on K_m. This suggests that Arg 180 and Glu 177 are not involved in substrate binding but in stabilizing the transition state of the catalyzed reaction.

Conversions of tyrosines 80 and 123 to phenylaline decrease activity roughly 10-fold whereas conversions to serine reduced activity roughly 100-fold. In each case Tyr 80 is seen to be slightly more important than Tyr 123, consistent with its stronger contact to the substrate ring. Other residues such as Asn 78, Asn 209, and Trp 211 have been mutated. They decrease activity by a factor of 10 or less, however, suggesting less important roles in catalysis.

A mechanism has been proposed for RTA action that incorporates both the structural and kinetic analyses of RTA. It is shown in **Fig. 4** (27, 34). The substrate binds in the cleft and adenine is sandwiched between tyrosines 80 and 123 in a stacking interaction. The leaving group adenine is protonated by Arg 180, promoting the C1'-N9 bond breaking and thereby forming oxycarbonium character on the ribose. This transition state structure is also stabilized by ion pairing with Glu 177. A water molecule, seen in the crystal structure, lies on the opposite side of the sugar ring from adenosine. It will be polarized by Arg 180 to a hydroxide character that rapidly attacks the sugar carbonium, completing the reaction.

During the structure/function analysis of RTA an interesting story emerged that serves as a cautionary tale about the interpretation of mutagenesis results. Conversion of Glu 177 to Ala (E177A) was seen to reduce activity about 20-fold, in contrast to the known importance of this residue. The X-ray structure of the mutant protein (37) confirmed an earlier hypothesis that the hole left by mutagenesis could be filled by a nonessential residue, Glu 208 (33). Glu 208 moves into the area normally occupied by Glu 177, and its carboxylate substitutes reasonably well in stabilizing the oxycarbonium intermediate. Data from the E177A mutation alone could have led to a misunderstanding of the role of this residue.

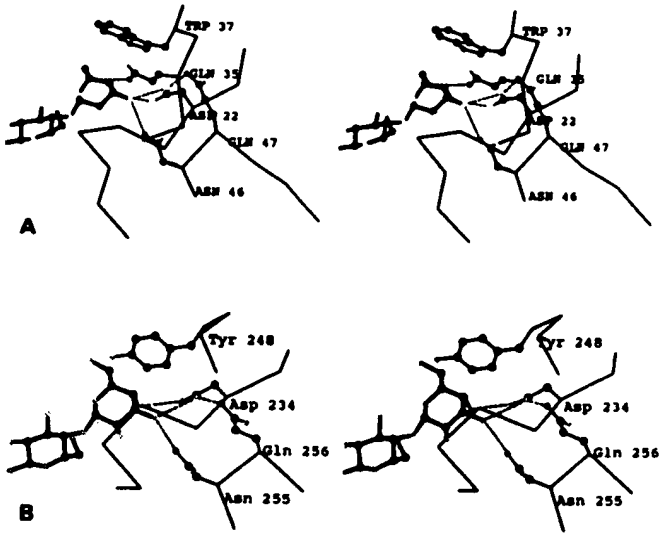

Figure 3. Lactose binding to ricin B chain. *A)* Lactose binding in the site on subdomain 1α. Hydrogen bonds are indicated as dashed lines between polar atoms. *B)* Lactose binding in subdomain 2γ.

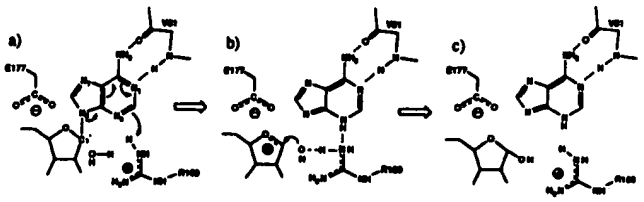

Figure 4. The mechanism of action for ricin A chain. *a)* Adenosine binds in the RTA active site. It stacks with the aromatic side chains of tyrosines 80 and 123 lying above and below. Specific hydrogen bonds are formed from the RTA backbone to the adenine ring as indicated by the dotted lines. *b)* The bond between adenine N9 and C1' of ribose is cleaved, aided by protonation of N3 by Arg 180. An oxycarbonium ion forms on ribose as the bond is broken, and this is stabilized by ion pairing to Glu 177. A water molecule on the opposite side of the ribose is hydrogen bonded to Arg 180. As Arg 180 transfers a proton to adenine, it removes one from the water and thereby activates it. *c)* The activated water attacks the oxycarbonium, releasing the adenine leaving group.

RICIN UPTAKE BY MAMMALIAN CELLS

Ricin enters cells by endocytosis, largely but not exclusively in coated pits, after opportunistically binding via its B subunit (RTB) to surface components containing terminal galactose residues. Potential surface binding sites,on both glycoproteins and glycolipids, are normally abundant: Hela cells, for example, possess 3×10^7 binding sites per cell for ricin. Mutant cell lines that are resistant to ricin often lack or contain only low levels of galactosyltransferase activity. After endocytic uptake of surface-bound toxin, RTB is believed to play a further role in that it facilitates, either directly or indirectly, delivery of the toxic A subunit (RTA) into the cell cytosol where its ribosomal substrate is located. Experimental findings pertaining to this have been interpreted in two different ways. The first was that distinct RTB sites or domains were responsible for surface binding and for the subsequent membrane translocation of RTA into the cell cytosol (38). The second proposed that the RTB galactose binding function was also required for the translocation function (39). This second interpretation has been proved correct. Recent work has shown that a ricin variant containing mutant RTB in which both galactose binding sites have been abrogated does not kill macrophages even though the mutant protein (itself a glycoprotein) can still bind to mannose receptors and is endocytosed by these cells. If one or another of the RTB galactose-binding sites is still active, the ricin mutant is potently cytotoxic even when binding to galactosides on the macrophage cell surface is prevented by the addition of free lactose (**Table 1**) (40).

Why should the RTB galactose binding function be required intracellularly for ricin cytotoxicity? Immunoelectron microscopy has shown that a portion of the endocytosed ricin is transported via endosomes to the trans Golgi network (TGN), suggesting that RTA translocation takes place from the TGN or a post-TGN compartment (41). With the exception of diphtheria toxin, which is known to translocate from acidified endosomes, several other plant and bacterial toxins appear to follow this route. Brefeldin A (BFA) treatment of cells protects against these toxins, although it does not block endocytosis nor does it prevent the toxins from reaching the TGN (42, 43). One simple interpretation of this data is that transport of the toxins into or through the Golgi stack (which is disrupted following BFA treatment) is essential before translocation into the cytosol can occur. Retrograde vesicular membrane flow through the Golgi stack to the ER has been recognized recently as a normal process in eukaryotic cells and one that counterbalances ongoing anterograde traffic. This backward flow allows the retrieval of ER luminal proteins that have escaped their normal residence. Retrieval involves a receptor that recycles between the ER and the Golgi and recognizes a tetrapeptide present at the COOH-terminus of soluble ER proteins. In mammalian cells this tetrapeptide is usually KDEL (44). Certain bacterial toxins, including *Pseudomonas* exotoxin A, contain a KDEL-like sequence at the COOH terminus that is essential for cytotoxicity, suggesting that these toxins may depend on interaction with the KDEL receptor for transport back through the Golgi stack and that translocation of the toxic polypeptide into the cytosol occurs from the ER lumen (45, 56).

Neither RTA nor RTB contains a COOH-terminal KDEL-like sequence, and this may explain the requirement for intracellular RTB galactose binding activity. Binding to galactose present on a recycling glycoprotein, which must have traveled the anterograde secretory route at least as far as the trans Golgi cisternae where galactosyl transferase is located, may be required to transport incoming ricin back through the Golgi, perhaps to the ER. This step may be very inefficient: only a small proportion of the ricin that reaches the TGN may proceed further along the retrograde pathway. The recycling proteins that putatively transport the toxin through the Golgi stack may themselves be carried by the KDEL receptor (47). For example, it is known that the ER lumen glycoprotein calreticulin is galactosylated, showing that it can be retrieved from the trans Golgi cisternae or beyond (48).

TABLE 1. *Cytotoxicity of ricin containing wild-type or mutant RTB towards Vero cells or peritoneal macrophages*[a]

	Cytotoxicity				
	Vero cells		Macrophages		
	Toxin alone	+ Lactose	Toxin alone	+ Lactose	+ Lactose and mannose
Wild-type	Yes	No	Yes	Yes	No
1st site mutant	Yes	No	Yes	Yes	No
2nd site mutant	Yes	No	Yes	Yes	No
Double mutant	No	No	No	No	No

[a]Ricin was reconstituted by reassociating wild type or mutant RTB produced in *Xenopus* oocytes with RTA produced in *E. coli*. The cytotoxicity of a range of protein concentrations was measured as [^{35}S]methionine incorporation into protein. The various forms of ricin are indicated as inhibiting protein synthesis completely (yes) or having no effect (no) at an equivalent concentration, and the effect of including lactose or a mixture of lactose and mannose on cytotoxicity is also shown.

The intracellular role of RTB in ricin cytotoxicity therefore may be to ensure delivery of RTA to a translocationally competent compartment, whereas the membrane translocation ability may be the exclusive property of RTA; however, the hypothesis is largely speculative at present. The potent cytotoxicities of many RTA-containing immunotoxins (ITs) (see below) and the cytotoxic enhancement seen when some ITs are administered to BFA-treated cells (49) suggest that alternative intracellular routes of entry may be available. Ricin variants require at least one of the two RTB galactose-binding sites to remain functional for cytotoxicity, even when the toxin binds to an alternative surface receptor such as the mannose receptor of macrophages (40). This suggests that ricin may change binding sites from the surface mannose receptor to an intracellular galactosylated receptor during intracellular transport, and that this change is essential for cytotoxicity. Similarly, *Pseudomonas* exotoxin is taken into cells by endocytosis after binding to the α_2-macroglobulin receptor (50) but may have to interact intracellularly with the KDEL receptor to reach the cytosol (45). Clearly, much more work is required to elucidate the pathway normally used by that fraction of the endocytosed toxin that ultimately enters the cytosol. Theoretically the ER is an appealing compartment for toxin translocation (containing protein disulfide isomerase, molecular chaperones, and the membranous machinery for the transport of proteins and peptides, all of which ricin might exploit (47)), but as yet there is no direct evidence that this organelle is involved. Alternative transport pathways and sites of toxin translocation should also be considered. For example, ricin may leave the Golgi in vesicles that normally transport lysosomal enzymes bound to the mannose 6-phosphate receptor and RTA may translocate to the cytosol from a prelysosomal vesicle.

THERAPEUTIC APPLICATIONS OF RICIN

Ricin is prominent among a group of toxic proteins that have been used in attempts to selectively kill unwanted cells, in particular, malignant cells. Delivery to the target cell is achieved by linking the toxin to an antibody or growth factor that specifically or preferentially interacts with the target cell in question. Ricin has generally been linked to monoclonal antibodies by a disulfide bond, formed using heterobifunctional cross-linkers. Such conjugates are called ITs. Occasionally ricin holotoxin has been conjugated to the antibody, but more often, to avoid nonspecific cell interactions mediated by RTB, the RTA subunit only has been used. Such conjugates display potent and specific cytotoxicity toward their target cells in vitro. However, when the same conjugates have been administered in vivo to tumor-bearing rodents or humans, the antitumor effects have often been disappointing (51).

In vitro applications

RTA-ITs have been used in vitro to purge bone marrow of unwanted cells prior to transplantation. In allogeneic bone marrow transplantation this has entailed using the IT to destroy T lymphocytes in the bone marrow taken from a histocompatible donor to reduce the incidence of graft-vs.-host disease (GVHD) in patients receiving the transplant (51). In autologous bone marrow transplantation, a sample of the patient's own bone marrow is removed before destroying the remainder. Before the patient can be successfully reinfused with the marrow removed previously, it must be free of malignant cells. Anti-T cell ITs have been used with some success to purge such bone marrow in the treatment of a variety of T cell leukemias and lymphomas (51).

In vivo applications

For in vivo use, RTA-ITs have generally proved more effective when the target cells are readily accessible to the bloodstream. In this regard, anti-T cell RTA-ITs have been the most successful, particularly in the treatment of steroid-resistant, acute GVHD (52). For the in vivo treatment of solid tumors, considerable problems can arise due to poor access of the IT to the tumor mass, lack of IT specificity, tumor cell heterogeneity, antigen shedding, breakdown or rapid clearance of the IT, and dose-limiting side effects. RTA-ITs cause vascular leak syndrome, resulting in hypoalbuminemia followed by weight gain and pulmonary edema (51). Repeated administration can be compromised by an immune response to both the antibody (often murine in origin) and the toxin components of the IT. Considerable effort has been and is being made to overcome these and other limitations. For example, early (or first generation) RTA-ITs contained RTA that had been chemically purified from *Ricinus* seeds. In addition to requiring rigorous purification procedures to completely exclude RTB contamination, the purified RTA was naturally glycosylated with mannose- and fucose-containing oligosaccharides. This allows the IT to interact with cells containing mannose and fucose receptors. In particular, the abundance of these receptors on liver cells led to the rapid hepatic clearance of the RTA-ITs. This problem was initially eliminated by chemically deglycosylating the RTA prior to IT construction (51), and more recently by the use of nonglycosylated recombinant RTA produced in *E. coli* (15). Recombinant DNA technology is increasingly used to produce recombinant ITs and other chimeric molecules for therapeutic use (46).

Although ITs have failed to live up to the high expectations initially placed on them and in spite of problems associated with their use at present, efforts currently under way should lead to further improvements and ensure that ITs will be increasingly useful as clinically important and selective cytotoxic regents.

TOXIGENIC ABLATION

Selective cell ablation is a useful way to investigate the developmental origin, fate, or function of particular cell lineages in an organism. Initially this was achieved by damaging or physically removing cells. Increasingly, toxigenic methods are being used that involve the expression of a toxic gene product in the target cells. This latter approach can overcome difficulties arising from either the failure to remove/destroy all target cells or general problems of cell accessibility that can sometimes limit the use of physical methods. Toxigenes are DNA fusions in which DNA encoding a potent toxin, such as RTA or a catalytically active fragment from a bacterial toxin such as diphtheria toxin, is placed under the transcriptional control of a tissue- or developmental stage-specific promoter and/or enhancer. When expressed intracellularly the toxigene product causes cell death. The introduction and expression of a toxigene in transgenic animals or plants may lead to cell type-specific ablation, which can be used to study developmental cell lineages or to generate animal models of degenerative diseases. Obviously, if the cell type selected for ablation is crucial for survival it would be impossible to derive genetically modified strains through breeding. When the cell lineage is not essential for survival,

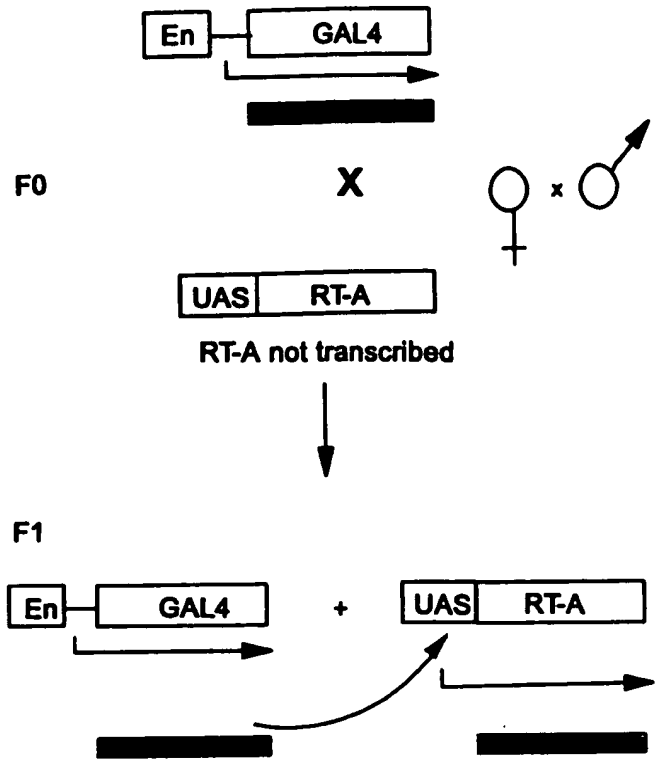

Figure 5. A two-component ablation system in which RTA is expressed under the control of a yeast GAL 4 upstream activation sequence (UAS). The toxin gene is only transcribed in the F1 progeny of the cross shown where GAL 4 expression under the control of a tissue-specific enhancer (En) drives the transcription of RTA by interacting with the UAS. (Figure courtesy of Dr. K. Moffat.)

the developmental effects of its ablation can be studied. The mammalian lens is such a system and transgenic mice carrying the RTA gene under the control of the α-A crystallin promoter showed lens abnormalities together with a malformation and abnormal development of the neural retina (53).

In an interesting recent development of this approach, conditionally mutant toxigenes that encode temperature-sensitive (ts) or cold-sensitive (cs) products have been used. This allows the timing of ablation to be selected in non-homeothermic organisms, where ablation can be triggered simply by performing a temperature shift from the restrictive temperature to the permissive temperature for toxin activity (54).

Two component ablation systems are also being developed in which the toxin is expressed only in the F1 progeny resulting from a genetic cross between two stable lines. One such system, which is currently used in *Drosophila*, involves crossing a line carrying a gene for the yeast transcriptional activator GAL4 with a line carrying the toxigene under the control of a GAL4-dependent upstream activation sequence (UAS) (55). In the GAL4 line, the GAL4 gene itself is under the control of a weak promoter that only becomes active when the fusion inserts into the host chromosome adjacent to an endogenous enhancer (**Fig. 5**). The availability of many enhancer-trap *Drosophila* lines with different GAL4 expression patterns ensures that a predicted toxigene expression pattern can be selected for the Fi progency. When this binary expression system also utilizes conditionally mutant toxigenes, ablation can be turned on at the desired stage of development as well.

CONCLUDING COMMENT

Ricin is an example of two component cytotoxic proteins in which a cell-binding component facilitates the delivery of a toxic component into the cell cytosol. Current interest should ensure that efforts to understand and utilize these interesting proteins continue. In particular, research will be directed toward identifying the intracellular site and mechanism of toxin translocation into the cytosol. FJ

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